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Isolation of a French bean (*Phaseolus vulgaris* L.) homolog to the β-glucan elicitor-binding protein of soybean (*Glycine max* L.)

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Abstract

A high-affinity membrane-bound β-glucan elicitor-binding protein has been purified from microsomal preparations of French bean (*Phaseolus vulgaris* L.) roots. A 5900-fold purification was achieved by affinity chromatography of functionally solubilized membrane proteins. The β-glucan-binding protein had an apparent molecular mass of 78 kDa when subjected to SDS-PAGE. Western blot analysis showed specific crossreactivity of this French bean protein with an antiserum raised against a synthetic peptide representing an internal 15 amino acid fragment of the β-glucan-binding protein from soybean. Northern blot analysis with a cDNA probe of the soybean β-glucan-binding protein gene revealed a crosshybridizing transcript of 2.4 kb in French bean. These results indicate that the β-glucan-binding proteins of French bean and soybean are conserved homologs involved in β-glucan elicitor recognition.

Keywords: β-Glucan elicitor-binding protein; Affinity purification; French bean

1. Introduction

Plants possess numerous defence mechanisms conferring resistance against potential pathogens including an oxidative burst, production of pathogenesis related proteins, and phytoalexin accumulation [1]. These defence responses can be induced not only by infection, but also by treatment of plant tissues with pathogen-derived compounds referred to as elicitors. This indicates the presence of specific recognition systems for the elicitors in host plants. Various substances of diverse chemical structures including components of the cell surface as well as excreted metabolites of oligosaccharide and (glyco)protein nature have been described as elicitors [2]. Progress in the isolation of pure elicitors has made possible biochemical investigations on elicitor-binding proteins which might function as receptors.

Treatment of the legume species, French bean, *Phaseolus vulgaris* L., with elicitors as well as infection with pathogenic fungi induced typical defence responses. Among the induced responses are immobilization of cell wall proteins [3], induction and accumulation of a polygalacturonase-inhibiting protein [4], and activation of defence genes and synthesis of phytoalexins [5–8]. Different elicitors, including carbohydrates, were used for these studies. In particular, a crude elicitor preparation from the cell walls of the phytopathogenic fungus *Colletotrichum lindemuthianum* [3], a linear 1,3–1,6-linked β-glucan [8], and a defined branched 1,3–1,6-β-glucan derived from the cell walls of the plant pathogen *Phytophthora sojae*

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[4,7] were used. The latter glucan was shown not only to induce defence responses in French bean, but also to bind with high affinity to a microsomal fraction of this plant [7]. This binding site inherited some of the properties that are typical for putative elicitor receptors [9,10], such as high affinity for the β-glucan ligands, low abundance, and correlation with biological function as reported for others.

In plants, besides biochemical characterization of several membrane-bound elicitor-binding sites, the identification of the binding proteins involved was reported in only three cases. By using photoaffinity-labeling and/or crosslinking techniques, in soybean three β-glucan binding proteins [11,12], in rice, one 75-kDa *N*-acetylchitooligosaccharide-binding protein [13], and in parsley, one 91-kDa tridecapeptide elicitor-binding protein were identified [14]. Up to now, only in soybean, was isolation of such a putative receptor successful. The β-glucan elicitor-binding protein of soybean was purified to apparent homogeneity using affinity-chromatography [12,15] and its cDNA cloned [15]. The present study describes the solubilization and purification of a β-glucan elicitor-binding protein from French bean. Immunological and Northern blot analyses provided evidence for the similarity between French bean and soybean β-glucan elicitor-binding proteins.

2. Materials and methods

2.1. Microsomal preparation and solubilization

French bean (*Phaseolus vulgaris* L. cv. Maravilla de Venecia) plants were grown in a phytochamber as described previously [7]. Roots from 14-day-old plants were used to prepare a total membrane fraction [16]. The microsomes were frozen in liquid nitrogen and used immediately or stored at −80°C. Membrane protein pellets were homogenized in 25 mM Tris/HCl, pH 8.0, 1 mM EDTA, 2 mM DTT, 20% (v/v) glycerol (buffer A) in a Potter Elvehjem with a Teflon pestle. Solubilization was started by adding 0.1% (w/v) Zwittergent 3-12 (dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propane-sulfonate, ZW 3-12; Sigma, Munich, Germany). After 1 h of gentle stirring on ice, the suspension was centrifuged at $150\,000 \times g$ for 45 min. The supernatant was collected and frozen in liquid nitrogen or precipitated with polyethylene glycol 4000 at a final concentration of 20% (w/v) on ice for 1 h. The suspension was centrifuged at $100\,000 \times g$ for 30 min. The pellet was dissolved in buffer A containing 0.1% (w/v) ZW 3-12 and used immediately or stored at −80°C.

2.2. Heptaglucoside-binding assay

Binding assays were performed according to Cosio et al. [16] using 30 nM ^{125}I-labeled 2-(4-aminophenyl)-ethylamine conjugate of the hepta-β-glucoside (HG-APEA). Synthesis of the HG-APEA was described earlier [17]. Non-specific binding was determined in the presence of a 1000-fold excess of unlabeled β-glucan. Protein content was measured according to Bradford [18] with bovine serum albumin as standard.

2.3. Synthesis of an affinity matrix and affinity chromatography

Synthesis of the β-glucan affinity matrix and all chromatography steps were carried out as described [12] with minor modifications. Briefly, solubilized membrane proteins (10 mg) in buffer A containing 0.1% (w/v) ZW 3-12 and 0.2 mM phenylmethanesulfonyl fluoride (PMSF) were loaded onto the β-glucan affinity column (2.5 ml bed volume). Using a peristaltic pump the sample was circulated for at least 15 h at 2°C at a flow rate of 0.5 ml/min. To remove non-specifically adsorbed proteins, the matrix was washed three times with 1.5 ml each of 25 mM Tris/HCl, pH 7.5, 0.2 mM PMSF, and 0.1% (w/v) ZW 3-12 (buffer B), and once with buffer B containing 1% (w/v) ZW 3-12. Glucan-binding proteins were eluted in two fractions by incubating the prewashed matrix twice with 1.5 ml each of buffer B containing 1 mg free β-glucan (90 min each). Fractions obtained after affinity chromatography were precipitated twice (1 h each) by adding polyethylene glycol 4000 to 20% (w/v) final concentration in the presence of 1 mg/ml aprotinin to remove the bound β-glucan [12].

2.4. Western blot analysis

SDS-PAGE was performed by using 10% separating and 3% stacking gels [19]. Immunoblotting ex-

periments were performed as described previously [12] using the anti-peptide antiserum, raised against an internal amino acid sequence of the soybean β-glucan-binding protein [12].

2.5. Northern blot analysis

Root total RNA from 5-day-old French bean plants was prepared according to Schröder et al. [20]. Northern blotting was performed according to standard protocols [21]. Degenerated oligonucleotides designed from internal peptides of the soybean β-glucan binding protein [12] were used for PCR amplification of soybean root cDNA. The obtained cDNA fragment (1500 bp) showed more than 99% sequence identity with the cDNA of the soybean β-glucan binding protein reported recently [15]. This partial cDNA fragment was used for random primed ^{32}P-labeling (Prime a Gene, Promega, Heidelberg, Germany) and hybridization according to [22].

3. Results and discussion

Although many elicitors of inducible plant defences have been characterized, only a few binding proteins which may function as receptors for elicitors have been identified by direct biochemical approaches [11,13,14]. But only in the case of the β-glucan-binding protein of soybean an elicitor-binding protein has been purified to homogeneity [12,15]. Very likely, this is mainly due to the low abundance of the binding proteins in plant cells and also to the fact that many of them are associated with membranes which causes problems during attempted purification.

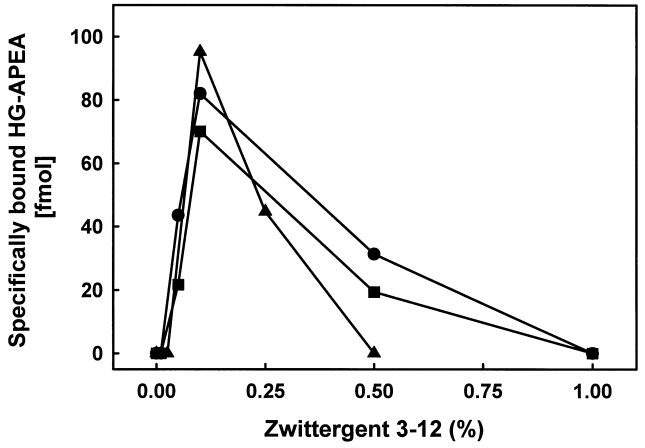

Fig. 1. Solubilization of β-glucan-binding proteins from microsomal membranes of French bean by the detergent Zwittergent 3-12. Following detergent extraction, the samples were centrifuged at 150 000×*g* for 45 min. β-Glucan-binding activity was determined in the supernatant. In the respective assays, the total specific HG-APEA binding to microsomal membranes without detergent was 35.3 fmol (▲), 34.9 fmol (●), and 23.3 fmol (■). For each data point, the non-specific binding was subtracted. Different symbols represent individual experiments.

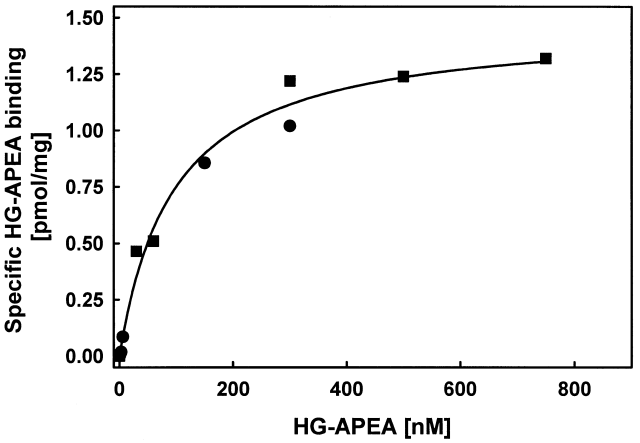

Fig. 2. Concentration dependence of specific ^{125}I-labeled HG-APEA binding to solubilized membrane proteins of French bean. The solubilizate was incubated with the indicated concentrations of ^{125}I-labeled HG-APEA. Data points show specific binding (total minus non-specific binding). Curve fitting and calculation of the binding constant were performed by non-linear regression. Different symbols represent different independent experiments.

Besides soybean, in microsomal fractions of French bean, the ability to bind β-glucan elicitor with high affinity was described [7], suggesting the presence of a β-glucan-binding protein. In order to use the affinity-chromatography technique for purification of the membrane-associated French bean protein(s), the prerequisite was their solubilization under conditions that preserve binding activity. Therefore, solubilization was carried out in the absence of the ligand and functionality of the binding protein was subsequently determined in the solubilized state by specific HG-APEA radioligand binding. The soluble nature of the binding activity in ZW 3-12 microsomal extracts was proved by lack of sedimentation at 150 000×*g* for 45 min. The optimum ratio for detergent to protein was determined to be 3:1 (w/w), when using a protein concentration of 1.5 and 2 mg/ml,

respectively. This protein to detergent ratio was nearly identical to that determined for the soybean β-glucan-binding protein [12,23]. In contrast to soybean, β-glucan-binding activity in French bean was found to be most efficiently solubilized around the critical micellar concentration (CMC) of ZW 3-12 (0.1%, w/v) and less efficiently at higher or lower detergent concentrations. Importantly, the final detergent concentration had to be kept at CMC to minimize loss of active binding protein (Fig. 1). Under these optimized conditions, the highest amount of microsomal radioligand-binding protein was extracted from membranes in functional form, as shown by HG-APEA radioligand saturation experiments (Fig. 2). The data showed saturability for HG-APEA with an apparent K_d value of 96 nM. This K_d value was three times higher than that determined for microsomal French bean proteins [7].

Only 70% of total membrane protein could be solubilized resulting in a 3-fold enrichment of specific binding activity (Table 1). Under these conditions, an increase in total binding activity was observed that might be due to an enhanced accessibility of binding sites that were initially cryptic in inside-out orientated membrane vesicles. Because of the modified experimental protocol, the resulting volume of the solubilizate was 10-fold higher when compared with that of the solubilization procedure used for soybean.

By exploiting the experience gained with soybean, the purification protocol was developed with a minimum number of steps in order to reduce activity losses. Therefore, affinity chromatography with immobilized β-glucans was chosen. To achieve a protein concentration of at least 1 mg/ml during chromatography, the sample was concentrated by polyethylene glycol 4000 precipitation prior to loading of the affinity column. After specific elution of binding activity from the affinity matrix with an excess of free β-glucan in the buffer, two enriched protein bands were visible on a silver-stained SDS-polyacrylamide gel with apparent M_r of about 78 000 and 91 000, respectively (Fig. 3B). The seemingly higher molecular weight of the French bean β-glucan-binding protein (78 kDa) in comparison with soybean (75 kDa) was demonstrated by an SDS-PAGE where both affinity-purified proteins were separated side by side on the same gel (Fig. 3E,F). The overall yield of the entire purification of the β-glucan-binding protein(s) from French bean roots was about 2% of the

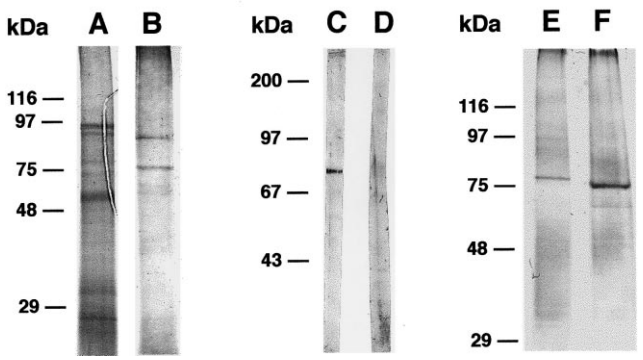

Fig. 3. SDS-PAGE and Western blot analyses of purified French bean β-glucan binding-proteins. (A,B) Silver staining of proteins after SDS-PAGE of crude solubilized and affinity-purified French bean membrane protein fractions. Portions of individual fractions obtained after affinity chromatography were precipitated and subsequently separated on SDS-PAGE (10% separating gel). Lanes correspond to crude solubilized membrane proteins (A; 15 μg) and eluate fraction from the affinity column (B) of which 3 ml were precipitated. Binding proteins were eluted from the affinity column with 1 mg β-glucan per fraction. (C,D) Western blot analysis of the purified β-glucan-binding proteins from French bean with anti-peptide a antiserum raised against an internal 15-amino acid sequence from the soybean β-glucan-binding protein. Binding proteins were purified by affinity chromatography, separated by SDS-PAGE (10% separating gel), blotted onto nitrocellulose membranes, and probed with anti-peptide a antiserum (C) or preimmunoserum (D), respectively, using final serum dilutions of 1:500. (E,F) Silver staining of proteins after SDS-PAGE of affinity-purified French bean (E) or soybean (F) membrane fractions (10% separating gel). From the eluate fraction 1 ml each was precipitated.

Table 1
Purification of β-glucan-binding proteins from French bean roots

Step	Protein (mg)	Total activity (pmol)	Specific activity (pmol/mg)	Enrichment (fold)
Membranes	143	22.9	0.16	1
Solubilizate	100	52.5	0.52	3.4
PEG precipitation	19	20.3	1.07	6.7
Affinity chromatography	0.0005	0.47	943	5890

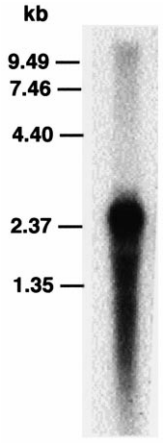

Fig. 4. Northern blot analysis of total RNA (25 μg) of French bean root tissue. The blot was probed with a radiolabeled PCR fragment of the soybean GBP. The positions of the fragment length standard (RNA ladder, Gibco-BRL) are shown on the left side.

original microsomal binding activity resulting in an increase in specific binding activity of about 5900-fold (Table 1).

To verify that one of the enriched proteins represented a β-glucan binding protein, we analyzed the crossreactivity with a specific antiserum raised against the 15-mer internal oligopeptide a of the β-glucan-binding protein from soybean [12]. This antiserum exclusively recognized a 78-kDa protein demonstrating the enrichment of a protein very likely related to the β-glucan-binding protein of soybean in the affinity-purified fraction from French bean membrane proteins (Fig. 3C). No crossreactivity was found with the preimmunoserum (Fig. 3D). With an antiserum raised against a 65-kDa polypeptide representing a putative subunit of the glucan synthase from French bean [24] also no crossreaction with any protein in the eluate fraction was found (data not shown). This indicated that the affinity-purified proteins do not represent the glucan synthase.

The presence and identity of a 78-kDa β-glucan-binding protein in French bean was further supported by a Northern blotting experiment. The radiolabeled soybean cDNA probe, representing a 1500-bp fragment of the soybean β-glucan-binding protein cDNA [15], showed crosshybridization with a transcript of about 2.4 kb in total root RNA of French bean (Fig. 4). The coding capacity of this mRNA is in good agreement with the molecular mass (78 kDa) of the bean binding protein (Fig. 3A) and with an mRNA of similar size encoding the soybean β-glucan-binding protein [15].

In conclusion, our results strongly suggest the existence of a French bean homolog to the β-glucan-binding protein of soybean, although both proteins seem to exhibit different binding characteristics. In particular, the soybean protein shows higher affinity to the hepta-β-glucoside elicitor than to other β-glucans. The reverse situation was described for French bean [7]. The different ligand-binding characteristics could be valuable to obtain information on the molecular structure and mode of action of these β-glucan elicitor-binding proteins. Obviously, within the legumes, these putative elicitor receptors possess high similarity concerning both their binding properties [7] and their molecular structure. Isolation and expression of the gene encoding the β-glucan-binding protein from French bean would allow a comparison of the two genes from French bean and soybean [15] and help to perform structure–function analyses. This offers, for the first time, the possibility of exploring a family of membrane-bound elicitor-binding proteins in plants.

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